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Studies on Malt Amylase. (III)

The Effect of Gibberellic Acid Treatment on the Alpha-Amylase Development in Wheat Seeds Pre-heated at High Temperatures.

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Gibberellin has a stimulatory effect on the germination and on the development of alpha-amylase (dextrinizing amylase) in cereal grains. The response of wheat seeds pre-heated at 100°C. (under dry) and at 60°C. (wet) to gibberellin was investigated. The stimulatory effect of gibberellin was altered by the heat treatment, and under certain conditions gibberellin did not serve as a stimulant at all. This suggested that the origin of alpha-amylase or factors affecting its formation promoted by gibberellin treatment might be different from those in natural germination, or the functions of exogenous and endogenous gibberellin on the development of alpha-amylase were not similar.

INTRODUCTION

In a classic study Brown and Morris[1], and Dickson and Shands[2] suggested that the embryo was the major source of hydrolytic enzymes involved in the degradation of the endosperm. Brown and Escombe[3] and Shands[4] indicated that aleurone was functional during germination. Swanson[5] and Ikemiya[6] demonstrated that amylase might develope in seeds without evidence of germination. Kirsop and Pollock[7] found that normal modification could occur in endosperm when embryo was removed after 2 or 3 days of growth. Paleg[8] and Yomo[9] demonstrated that gibberellic acid was capable of inducing the formation or activation of alpha-amylase in de-germed barley seeds.

Chrzaszcz and Janicki[10] suggested that alpha-amylase existed in the resting seed in a latent form, from which it was released by the action of eleuto-substances. Studies on barley by Briggs[11] supported the concept of alpha-amylase synthesis. Briggs[12] has estimated for germinated barley that the embryo accounts for 7% of the total alpha-amylase while 93% is found in the endosperm. Strivasta and Meredith[13] and Harris[14] reported that alpha-amylase elaboration was inhibited if a protein synthetase inhibitor was present.

Gibberellic acid, one of nine closely related compounds known as gibberellins, has a profound effect upon malt and other growing plants. Since Hayashi[15] demonstrated that gibberellin stimulated formation of amylase in barley and wheat in 1940, numerous workers have studied the response of cereal grains to treatment with gibberellin during malting process. However, these studies were performed on intact and viable seeds. The effects on cell free systems and non-viable seeds have been rarely studied[16].

It is quite well known that retention of viability of the heated seeds increases with

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decreasing moisture content and the proteins are less susceptible to denaturation by heat in the absence of moisture. The effect of heat treatment on seeds at higher temperatures has been studied by several investigators.

This work was carried out to obtain information on the origin of alpha-amylase in wheat seed, and heat treatment was utilized to inhibit the germination.

EXPERIMENTAL

Triumph (hard red winter) wheat (1962 crop) and Omar wheat (1961 crop) which had been stored in cold room (4°C.) were used in the experiment in 1964.

Potassium gibberellate (GA) employed was a product of Merck & Co., Rahway, New Jersey, U.S.A..

Roccal (10% solution of alkyl dimethyl benzyl ammonium chloride) was purchased from Sterwin Chemical, Inc., N.Y., U.S.A..

The wheat seeds were surface-sterilized after steeping with GA and water, with 0.1% Roccal solution for 5 minutes at room temperature and washed with water. The steeped seeds were incubated on a moist filter paper in covered petri dishes placed in a germination cabinet. A relative humidity of 76% and a temperature of 17°C. were maintained during incubation. After the incubation, malts were kilned in a forced convection oven (40°C.), ground and extracted with 0.2% calcium chloride solution. The acitivty of alpha-amylase (SKB-unit) in the extract was determined by the procedure of Sandstedt, Kneen and Blish[17]. SKB-units per g. were reported on a moisture free basis.

Moisture content was determined by the two stage air oven method[18] and was expressed on a wet weight basis.

Germination ratios represented the percentages of seeds in which emergence of both rootlet and sprout occured except for the sample which had a moisture content of 13.75% and heated for 4 hours in Table 4. Lengths of rootlet and sprout of germinated seeds were expressed as an average of 50 kernels.

Malted wheat seeds contaminated with fungi were detected by microscopic examination and then removed from the sample.

RESULTS AND DISCUSSION

The effect of wet heating is shown in Table 1. For these experiments, 10 g. of Triumph wheat seeds were kept in 200 ml. of 0.1% $CaCl_2$ in water bath at 55° and 60°C.. The seeds were subsequently steeped with 25 ml. of water or 0.001% GA solution for 20 hours at room temperature (23°C.).

It is evident from these data that heat-treatment of the moist seeds was critical. Even heating for 15 minutes at 55°C. greatly inhibited germination and the subsequent development of alpha-amylase which would be essentially restored if the heated seeds were soaked in GA (0.001%). Increasing the length of heating time at 55°C. or increasing the temperature to 60°C. decreased the alpha-amylase development accordingly. If the wheat was heated at 60°C. for 30 minutes, no alpha-amylase activity could be detected upon incubation for 5 days unless the wheat was treated with GA.

Table 1. Effect of GA on seeds pre-heated at 55° and 60°C.

Steeping with	Incubation	Malt	Non-heated Control			Heat-treatment								
						55°C. 15 min.			55°C. 30 min.			60°C. 15 min.		
			G.	A.A.	A.R.	G.	A.A.	A.R.	G.	A.A.	A.R.	G.	A.A.	A.R.
	Days		%	SKB	%	%	SKB	%	%	SKB	%	%	SKB	%
Water	3	germ. ed	98	47.2	100	62	6.8	100	13	0.2	100	7	5.1	100
		non-germ. ed		0	—		0	—		0			0	
GA	3	germ. ed	98	76.0	161	63	31.7	466	18	3.6	1800	8	17.0	333
		non-germ. ed					24.0	353		3.0	1500		15.9	312
Water	5	germ. ed	98	83.0	100	65	18.5	100	15	1.1	100	9	12.3	100
		non-germ. ed		0	—		0			0			0	
GA	5	germ. ed	98	175.3	211	70	97.6	528	20	17.6	160	11	68.3	555
		non-germ. ed		0	—		83.1	449		15.2	130		64.1	521

Abbreviation
G.=Germination ratio (%). A.A.=Alpha-amylase activity (SKB units). A.R.=Alpha-amylase activity ratio, GA-steeped/Water-steeped. Activity in water-steeped sample is presented as 100. germ. ed=germinated. non-germ. ed=non-germinated.

Even so, the alpha-amylase activity was so low. These data suggest that the mechanism related to germination and growth are more heat sensitive than the mechanism responsible for alpha-amylase development.

Calcium ion is considered as an activator and a stabilizer of alpha-amylase. To investigate the effect of the calcium ion on heat treatment of seeds, the Triumph seeds were treated at 55° and 60°C. for 15 minutes with various concentrations of $CaCl_2$ solution. The seeds were resteeped with 0.001% GA for 20 hours at room temperature and incubated for 3 days. The results are shown in Table 2.

Table 2. Effect of concentration of calcium chloride in the heat-treamtent on alpha-amylase deveolpment in seeds.

Calcium-chloride	Heat treatment	
	55°C., 15 min.	60°C., 15 min.
%	SKB-units/g	
0 (control)	66.0	3.6
0.01	66.5	7.3
0.05	70.2	12.8
0.10	76.8	16.4
0.25	81.0	20.6
0.50	65.7	10.5
1.00	63.5	9.6
2.00	52.0	4.3

On the heat treatment of the seeds, 0.25% $CaCl_2$ appeared to be optimal. However, since alpha-amylase is not present in the active form during heat treatment, calcium ion may protect the precursor of alpha-amylase from heat denaturation.

In contrast to wet heating, the effect of dry heating was investigated. The

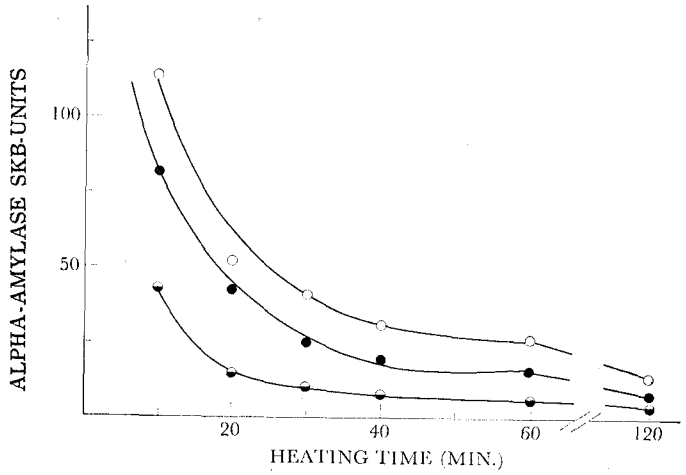

Fig. 1. Effect of GA treatment on alpha-amylase develpoment in seeds pre-heated at 100°C.. -○—○- germinated seeds steeped with GA, -●—●- germinated seeds steeped with water, -◒—◒- non-germinated seeds steeped with GA.

effect of heat treatment at 100°±1°C. on alpha-amylase development in seeds are presented in Fig. 1. Triumph seeds (moisture 12.4%) were kept at 100°±1°C. in hot-air oven for the time shown in Fig. 1. The seeds were subsequently steeped with water or 0.001% GA solution for 20 hours at room temperature (24°C.). Amylase activities of seeds incubated for 8 days at 17°C. are shown. Ninety-two % of the seeds heated for 10 minutes germinated, but only 37% of those heated for 120 minutes were viable. The decrease of alpha-amylase development in the seeds was rapid up to 30 minutes of heating time. The moisture content decreased from 12.4 to 8.9% during this heating time. After that time, the gradual decrease continued up to 120 minutes of heating time. Treatment of the heat-treated seeds with GA caused a slight recovery of the alpha-amylase activity.

The other series of experiments using dry-heat treatment was performed to obtain more information on the mechanism of alpha-amylase develpoment. Omar wheat seeds containing 9.4% moisture were dried in air convection oven for 40 hours at 35°C. in order to reduce the moisture content to 5.76%. One or two ml. of water was added to 40 g. of seeds (moisture 9.4%) and the seeds were stored for 21 hours at room temperature (20–22°C.) to obtain an increased moisture content of 11.61 or 13.75% of these seeds. The seeds were thinly spread over a tin plate and heated at 100°±1°C. for 1 to 4 hours. After this heating, the seeds were kept at room temperature for 2 hours. Ten g. of seeds pre-heated were steeped with 25 ml. of water or 0.001% GA solution for 20 hours at room temperature. The steeped seeds incubated for 3 or 7 days as described in the Experimental.

The effect of heat treatment at 100°C. for 1 to 3 hours on the germination and development of alpha-amylase is shown in Table 3. The seeds (of 9.4% moisture) used in the experiment probably had moisture low enough to prevent damage of

Table 3. Effect of heat treatment at 100°C on alpha-amylase development in wheat.*

Heating	Steeping with	Germination	Mean length of root	Mean length of sprout	Alpha-amylase activity	Alpha-amylase activity increased
hour		%	mm		SKB-units	%
			after 3 days incubation			
0	Water	98	6	12	63.7	—
	GA	98	8	15	103.2	62.0
1	Water	98	6	8	32.2	—
	GA	98	6	8	60.0	86.3
2	Water	95	6	7	18.5	—
	GA	95	6	8	24.0	29.7
3	Water	87	5	6	14.2	—
	GA	85	5	6	15.0	5.6
			after 7 days incubation			
0	Water	98	15	30	147.2	—
	GA	98	20	33	207.4	40.9
3	Water	87	16	21	37.6	—
	GA	85	20	22	37.6	0

* Original moisture content 9.41%

embryo occured by heating at 100°C. for 1 to 3 hours. French[19] found that heating at 85°C. up to 48 hours had little effect on the germination percentage of barley seeds. The author demonstrated that the germination ratio and development of alpha-amylase decreased with increasing time of heat treatment in triumph wheat seeds (of 12.4% moisture) in Fig. 1. From the data in Table 3 it is evident that the heat treatment decreased both development of alpha-amylase and stimulatory effect of GA. The increase of alpha-amylase caused by GA was 62.0, 86.3, 29.7 and 5.6% for 0, 1, 2 and 3 hours pre-heating of seeds, respectively. Alpha-amylase activity after 7 days of incubation was probably at a maximum for the germinated seeds, and therefore the maximum activity of these seeds pre-heated for such periods were 25.5 and 18.1% of activities of non-heated and water-steeped seeds, and of GA-steeped ones, respectively.

The viability of the embryos was not damaged by the heat treatment. It is considered that most of the alpha-amylase in malt is located in the endosperm and originated in aleurone layer[12]. The heat treatment probably denatured some precursors and/or enzyme systems in the aleurone cells concerned with the development of alpha-amylase. The data of Table 3 suggested that GA was not always stimulatory to germination and development of alpha-amylase in viable seeds.

The data in Table 4 show the relationship between moisture content in seeds and heat treatment at 100°C.. The seeds of 13.75% moisture, pre-heated for 4 hours, had the lowest germination ratio and showed the emergence of only the sprouts. Control (non-heated) seeds of different moisture contents showed almost the same modification and alpha-amylase development. It is of interest that seeds pre-heated at 100°C. for 4 hours were still viable whether with or without the addition of GA.

Table 4. Effect of moisture content and heating at 100°C. on alpha-amylase development.

Moisture	Heating	Steeping with	Germination	Mean length of root	sprout	Alpha-amylase activity	incressed
%	hour		%	mm.		SKB-units	%
5.76	0	Water	98	5	10	66.3	—
		GA	98	6	12	105.2	58.7
	2	Water	97	5	8	52.4	—
		GA	99	6	9	76.5	46.0
	4	Water	84	4	7	28.2	—
		GA	86	5	7	34.1	20.9
9.41	0	Water	98	6	12	63.7	—
		GA	98	8	15	103.2	62.0
	2	Water	95	6	7	18.5	—
		GA	95	6	8	24.0	29.7
	4	Water	80	4	5	13.1	—
		GA	83	4	5	13.1	0
11.61	0	Water	96	5	8	61.1	—
		GA	98	7	12	102.5	67.8
	2	Water	82	5	7	18.2	—
		GA	80	6	8	19.3	6.0
	4	Water	61	3	4	8.1	—
		GA	64	4	4	10.2	26.0
13.75	0	Water	96	5	9	61.6	—
		GA	99	7	12	100.4	63.0
	2	Water	78	4	1	9.0	—
		GA	81	4	1	18.2	102.2
	4	Water	40	2	0	3.1	—
		GA	64	2	0	7.5	141.9

Ben-Zeer and Zamenof[20] found that 70% of barley seeds with low moisture level pre-heated at 120°C. for 16 minutes still germinated. Alpha-amylase formation in seeds decreased as the moisture content was increased and as the time of pre-heating was increased. The stimulating effect of GA on alpha-amylase development in seeds was decreased by the pre-heating, except in the case moisture content of seeds was 13.75%, and the stimulatory effect of GA was increased by pre-heating. Modification of germinated seeds pre-heated for 2 hours was almost identical for moisture contents of 5.76, 9.41 and 11.6%, although the alpha-amylase development in the latter two samples was about 1/3 as much as that of the lowest moisture level.

As shown in Table 1 and 2, the wheat seeds treated with hot water destroyed the germinating ability within a short time. The alpha-amylase development, however, was greatly stimulated by GA treatment. Therefore, it is suggested here, that the factors associated with alpha-amylase development stimulated by GA are more thermostable than those factors associated with germination *per se*. The data presented here indicate that under dry heating, the factors and parts of the seed related to alpha-amylase formation are more thermolabile whereas those related to

germination (in germ part) are thermostable.

In order to determine the thermostability of alpha-amylase in malted seeds, dried seeds (moisture content of 9.7%) which were pre-steeped with or without GA and incubated normally were kilned for 3 hours at 100°C.. The remaining alpha-amylase activity after the heat treatment was 63.6 and 76.5% of initial activity in water-steeped and GA-steeped malt, respectively. Comparing these results with the data of Table 3, the factors concerning with the formation of alpha-amylase during germination are probably much less thermostable than the alpha-amylase itself developed in wheat seeds.

Paleg[8] found that barley seeds pre-heated at 100°C. (dry) for 1 hour showed less response to 2 and 200 ppm. GA than to 0.2 ppm. of GA in incubated seeds. Ben-Zeer and Zamenof[20] demonstrated that the stimulatory effect of GA of the germination of seeds heated at 120°C. was higher than those heated at 115°C.. Their data also suggested that pre-heating at higher temperature altered response of seeds to GA.

The present data showed that stimulatory effect of GA on the development of alpha-amylase was lost through certain heat treatments. These data suggest: (1) Seed parts or factors associated with the development of alpha-amylase which may be stimulated by the heat treatment with or without GA are not identical to each other. (2) The function of endogenous GA on germination may not be similar to the function of exogenous GA and be available under the conditions where exogenous GA is not available. Lazer, Baumgartner and Dahlstrom[21] demonstrated that GA occuring naturally in germinated barley seeds was probably in a bound or derivative form. (3) The heat treatment used in malting may be applied to obtain the malt of low alpha-amylase activity.

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